**Chronic Hepatotoxicity of Carbon Tetrachloride in HSP-70 Knock Out Mice**

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**Abstract:** The chronic hepatotoxic effects of carbon tetrachloride (CCl₄) in heat-shock protein (HSP) 70 knock out (HSP70−/−) mice were examined. After repeated intraperitoneal injections of CCl₄ for six weeks, the level of ALT and weight ratio of the liver to body were lower in HSP70−/− mice than in the control (WT) mice. The levels of HSP25 and HSP47 were lowered in HSP70−/− mice as compared with WT mice. The grades of hepatic necrosis and neutrophil infiltration were not significantly different between HSP70−/− and WT mice. The collagen content was not affected significantly by CCl₄ treatment. **Key words:** CCl₄, chronic hepatotoxicity, HSP-70, knock out mice

Chemically induced liver damage is a major toxicological problem. CCl₄ induced liver cell necrosis followed by regeneration is caused by free radical damage and may be partly due to oxidative stress and the subsequent formation of reactive oxygen intermediates [12]. The free radical products then induce an inflammatory response through the activation of Kupffer cells, resulting in liver cirrhosis.

Heat shock proteins (HSPs) are known to be crucial for activating cytoprotection mechanisms against xenobiotic exposure, although the mechanism remains equivocal. Oxidative stress directly triggers synthesis of HSP70 in centrilobular hepatocytes to counter lipid peroxidative damage. The close relationship between oxidant stress and HSP70 mRNA suggests that the binding of HSP70 protein to other damaged proteins reduces the availability of HSP70 protein, thereby inducing HSP70 expression universally under stress conditions [8]. CCl₄ treatment increased HSP70 and HSP25 levels in murine livers [9]. Thus HSP70 and other stress proteins seem to be important for preventing the accumulation of aberrant proteins. Further, the elevation of HSPs was reported to result in cytoprotection from a variety of subsequent stresses [3]. The binding of HSP70 to damaged proteins is believed to assist in preventing their aggregation and promoting correct refolding (“molecular chaperoning”), as well as facilitating their degradation [1].

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This study investigated the role of HSP70 in the defense mechanism of the liver of HSP70–/– mice chronically exposed to CCl4.

The HSP70–/– mice [4] and wild type (WT; C57BL/6) mice were obtained from the Cancer Research Center of Seoul National University. The animals were maintained in an SPF barrier system with controlled lighting (07:00–19:00), temperature (23 ± 2°C), relative humidity (55 ± 5%) and ventilation (10–12 times per hour).

Chronic liver damage was produced by injecting the following doses of CCl4 in olive oil intraperitoneally twice per week, 0.3 ml/kg in the first week, 0.7 ml/kg in the second week and 1 ml/kg for the next six weeks. The mice were sacrificed 48 h after the last administration and the body weights (BW) and liver weights (LW) were measured. Serum was stored at –80°C for analysis of alanine aminotransferase (ALT) [13]. Two slices of liver were fixed in 10% buffered neutral formalin for collagen analysis, and the rest of the liver was stored at –80°C after freezing in liquid nitrogen. Portions of the liver were then used for histology and for protein extraction. Necrotic lesions of liver slices stained with hematoxylin-eosin (HE) were scored histologically as follows: 0 = absence; 1 = minimal, spotty necrosis; 2 = moderate nonconfluent necrosis; 3 = submassive confluent necrosis; 4 = massive necrosis. Neutrophils were counted on 10 fields (400 ×) of each liver slice, centered on a centrilobular vein [13].

Liver collagen was quantitatively analysed by morphometry with formalin-fixed tissues, using Marielle Gascon-Barre methods [6]. On two tissue sections approximately 15 µm thick, 50–100 mm2 adjacent areas were studied morphometrically.

Frozen tissue was homogenized in 500 µl of lysis buffer (20 mM Tris, 137 mM NaCl, 10% glycerol, protease inhibitor). The lysate was then centrifuged at 9,000 g for 20 min and the supernatant fraction was collected. The protein concentration was determined by BCA protein assay. Aliquots of 30 g were diluted in SDS sample buffer, boiled and run immediately on 12% acrylamide SDS-PAGE gels. Proteins were transferred electrophoretically to nitrocellulose membranes, and the membranes were blocked in 5% skim milk/Tris buffered saline and reacted with primary antibodies (1 g/ml in each case) to HSP25 (SPA-801), HSP47 (SPA-470) and HSP70 (SPA-810) for 2 h in Tris-buffered saline plus 1 mg/ml of BSA. After washing three times with Tris-buffered saline containing 0.1% Tween-20, the membranes were treated with peroxidase conjugated secondary antibodies (1:5,000) and visualized using a Enhanced Chemical Luminescence (ECL).

Statistical comparisons were made using the two-tailed Wilcox or Mann-Whitney U tests.

Irrespective of the CCl4 treatment the LW/BW ratio in HSP70–/– mice was significantly lower than in WT mice (Fig. 1). The degree of hepatic necrosis and mean number of neutrophils were not significantly different between HSP 70–/– and WT mice (Table 1). No significant discrepancy was seen in the mean number of PCNA.

In the CCl4 treated HSP70–/– mice ALT was slightly lower than in WT mice, while non-treated HSP70–/– mice showed slightly higher ALT values than WT mice. There was no significant difference in ALT values be-

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**Table 1.** Degree of liver necrosis in HSP–/– and WT mice after CCl4 treatments

<table>
<thead>
<tr>
<th>Degree of necrosis</th>
<th>HSP70–/–</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>4 (20%)</td>
<td>3 (15.6%)</td>
</tr>
<tr>
<td>2</td>
<td>14 (70%)</td>
<td>14 (73.7%)</td>
</tr>
<tr>
<td>3</td>
<td>2 (10%)</td>
<td>2 (10.7%)</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>20 (100%)</td>
<td>19 (100%)</td>
</tr>
</tbody>
</table>

* Number of mice showing the degree of necrosis with % of test mice in parenthesis.
tween CCl₄ treated and control groups (Fig. 2). With Western blotting the expressions of HSP25 and 47 on liver proteins were shown to be decreased effectively after chronic exposure to CCl₄, but no significant change was seen in the values of HSP60 and HSP90 in HSP70–/– mice. In contrast, HSP25 and HSP47 remained unchanged, and HSP60 and HSP90 were amplified by CCl₄ treatment in WT mice (Fig. 3). As shown in Fig. 4, no significant change in the collagen level was shown following the chronic CCl₄ treatment in HSP70 knock out.

CCl₄ induced cirrhosis is known as an appropriate model of hepatic cirrhosis. Prokaryotic and eukaryotic cells respond to a variety of stresses resulting in the transcription of a specific set of genes that encode heat shock proteins [11]. Induction of HSPs under physiological conditions provides substantial protection against some hepatic toxicants including CCl₄ [7] and many studies have focused on the induction of HSP70. CCl₄, a xenobiotic, activates the cytochrome P450 mechanism, and the resulting free radicals contribute to the occurrence of oxidative stresses such as lipid peroxidation, which are known to affect directly HSP. Particularly, HSP70 has a pivotal role in inducing a defense mechanism in the irritated liver.

The expression of the stress inducible HSP70 gene was reported to increase transiently at the early stage of CCl₄ treatment following a transient increase in the expression of the early response genes *c-fos* and *c-jun* [12]. HSP25 is associated with increased resistance or tolerance to hepatotoxicants in the company of HSP70 [7]. Besides, HSP47, a collagen-binding stress protein, is assumed to act as a collagen-specific molecular chaperone during the biosynthesis and secretion of pro-collagen in the living cells [2]. In the liver of CCl₄-treated or bile duct-ligated mice, the number of HSP47-positive cells markedly increased in the centrilobular or around the periportal area, showing an increased level of HSP47 [2]. The present study revealed that the level of HSP25 notably diminished while the expression of HSP47 was reduced in HSP70–/– mice, suggesting that the chronic CCl₄ exposure decreased the liver fibrosis. This is compatible with the slightly lower collagen content in HSP70–/– mice than in WT mice after chronic administration of CCl₄.

![Fig. 2. Serum ALT levels in HSP70–/– and wild type (C57/BL6) mice. ALT level was elevated after CCl₄ treatment without significant difference between HSP70–/– and wild mice.](image)

**Fig. 2.** Serum ALT levels in HSP70–/– and wild type (C57/BL6) mice. ALT level was elevated after CCl₄ treatment without significant difference between HSP70–/– and wild mice.

![Fig. 3. The HSP density in western blots HSP47 is significantly lower in HSP70–/– mice than in WT mice. HSP60, 70 and 90 were not diminished even after CCl₄ treatment (*: means P<0.05).](image)

**Fig. 3.** The HSP density in western blots HSP47 is significantly lower in HSP70–/– mice than in WT mice. HSP60, 70 and 90 were not diminished even after CCl₄ treatment (*: means P<0.05).
Acknowledgments

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References


Fig. 4. Collagen contents in WT mice and HSP70–/– mice after CCl4 treatment (*: means P<0.05).

The induction of resistance and tolerance to hepatotoxicity results from the combination of the expression of HSP70 with those of other HSPs, such as HSP25 and HSP47. In HSP70–/– mice the HSP47 levels are overexpressed, and the ALT value is higher than in WT mice after acute exposure to CCl4 (unpublished observation). Some other defense mechanisms to hepatic toxicants might also be activated in the absence of HSP70.

The degree of hepatic necrosis, neutrophil infiltration and PCNA labeling index, level of ALT value and collagen content were not significantly different between HSP70–/– and WT after chronic administration of CCl4, indicating that the absence of HSP70 has no effects on hepatic fibrosis and regeneration after chronic CCl4 exposure.